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In addition to its function in virus assembly, the viral matrix (M) protein of vesicular stomatitis virus (VSV) inhibits host-directed gene expression. The goal of this study was to determine whether sequence changes in M protein contribute to a reduced shut off of host gene expression in cells persistently infected with VSV. Viruses isolated from L cells persistently infected with VSV inhibited host RNA synthesis more slowly than wild-type (wt) VSV. M genes of the persistent viral population were cloned and sequenced. One mutation, an N to D change at position 163 of the protein sequence (N163D), was common to all the molecular clones. The N163D M protein was synthesized from transfected mRNA at a rate that was 30% of that of wt M protein, but was turned over at a rate that was similar to that of wt M protein. Transfection of mRNA encoding N163D M protein inhibited expression of a cotransfected target gene encoding chloramphenicol acetyl transferase (CAT), but the inhibition was 6 to 10 times less effective than transfection of equivalent amounts of wt M mRNA. This difference could not be accounted for by differences in translation of CAT mRNA. Thus, when the differences in M protein expression were taken into account, N163D M protein was 2 to 3 times less effective than wt M protein in the inhibition of host-directed gene expression, similar to the differences in host transcription observed in virus-infected cells. Point mutations in addition to the N163D mutation were found in about half of the M gene molecular clones. The M gene of an independently isolated molecular clone, N163D.2, contained two additional point mutations in its carboxy terminal region. N163D.2 M protein was highly defective in inhibition of host gene expression and was turned over more rapidly than wt M protein. These results support the idea that M gene mutations contribute to a reduced cytopathic effect in cells persistently infected with VSV.

INTRODUCTION

VSV infection induces a spectrum of cytopathic effects (CPE), ranging from a rapid and potent inhibition of host macromolecular synthesis which affects cellular RNA, DNA, and protein synthesis, to the disruption of the host cell architecture leading to cell rounding (reviewed by Wagner, 1987). The matrix (M) protein of VSV is a major structural protein that normally functions in virus assembly by binding the viral nucleocapsid to the cytoplasmic surface of the host plasma membrane during budding (reviewed by Lenard, 1996). In addition to its role in virus assembly, VSV M protein is also involved in the cytopathic effects of virus infection. M protein causes the cell rounding characteristic of VSV infection (Blondel *et al.,* 1990). In addition, M protein is capable of inhibiting host-directed gene expression *in vivo* independently of other viral components (Black and Lyles, 1992; Black *et al.,* 1993; Paik *et al.,* 1995). The inhibition of host-directed gene expression by M protein occurs at the level of host transcription (Black and Lyles, 1992). However, host-directed translation is not inhibited when M protein is expressed in the absence of other viral components (Black *et al.,* 1994). Instead, the translation of cotransfected mRNAs is enhanced by expression of M protein (Black *et al.,* 1994). Thus, M protein has multiple effects on host expression, the overall result of which is a potent inhibition. It has been estimated that only 10,000 copies of M protein per cell are required to inhibit host-directed gene expression (Lyles *et al.,* 1996).

The function of M protein in inhibition of host gene expression is genetically separate from its role in virus assembly. This was demonstrated by using the M protein of the conditionally temperature-sensitive mutant of VSV, tsO82 (Coulon *et al.,* 1990). TsO82 virus contains a single point mutation in its M gene causing a methionine to arginine substitution at position 51 of the protein sequence. TsO82 M protein was fully functional in virus assembly as demonstrated by complementation assays, but was defective in its ability to inhibit host gene expression (Black *et al.,* 1993; Kaptur *et al.,* 1995). However, a second mutant, MN1, which lacked the positively charged amino-terminal region (spanning amino acids 4 to 21) of M protein displayed full activity in inhibition of host gene expression but its ability to function in virus assembly was abolished (Black *et al.,* 1993).

Persistent infections are generally characterized by a reduction in the typical cytopathic effects of virus infection. However, infectious virus continues to be produced.

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Studies have shown that persistent VSV infections result in the spontaneous selection of temperature sensitive (ts) viruses (Youngner *et al.,* 1976). The viruses isolated from persistently infected cells remain cytopathic when inoculated into fresh cells but the cytopathic effect is delayed as compared to wt VSV infections (Frey and Youngner, 1984). Virus clones isolated from persistently infected cells are able to establish new persistent infections when added to cells at low multiplicities of infection (m.o.i.) (Youngner *et al.,* 1976). Both cellular and viral factors appear to play a role in the maintenance of persistent VSV infections. The persistent state appears to be maintained by a low level of interferon (Youngner *et al.,* 1978). Therefore, balance between the ability of the virus to shut off host cell functions versus the ability of the host defense system to combat virus infection is required to maintain the persistent infection.

Since the involvement of VSV M protein in virus-mediated cytopathic effect is genetically distinct from its role in virus assembly, this raises the possibility that mutations in the M gene of VSV that interfere with host shut off functions without altering assembly functions contribute to the establishment of persistent viral infections. Several negative strand RNA viruses are known to establish persistent infections. Altered measles M mRNAs resulting from cellular RNA unwinding and modification activity have been found in the brains of patients with subacute sclerosing panencephalitis (SSPE), a persistent neurological disease due to measles virus infection (discussed by Wong *et al.,* 1991). In this disease, a whole spectrum of phenotypes are found, including an absence or reduced amount of M protein due to rapid turnover of the protein (Young *et al.,* 1985). Furthermore, in BHK cells persistently infected with Sendai virus, the level of viral M protein was reduced as a result of enhanced turnover of M protein (Roux and Waldvogel, 1982). These studies implicate mutant M proteins in the establishment or maintenance of persistent viral infections. In these cases, it was presumed that the effect of M gene mutations was to attenuate the yield of infectious virus. However, in the case of persistent VSV infections, M gene mutations could have the additional effect of reducing virus-induced CPE.

To determine whether VSV M protein mutations associated with persistent infections contribute to a defective inhibition of host-directed gene expression, a virus population from L cells persistently infected with VSV was analyzed. Viruses from this line have been well characterized and are less effective than wt VSV in inhibition of host RNA and protein synthesis, indicating that viruses isolated from persistently infected cells have a reduced cytopathic effect as compared to wt VSV (Frey and Youngner, 1984). M genes were cloned from the viruses from persistently infected cells, and sequence results revealed that the virus population from these cells contained a heterogeneous M gene pool with one conserved mutation at position 163 of the protein sequence (N163D). Analysis of this mutation in the wt background indicated that it inhibited host-directed gene expression, but was less effective than wt M protein. Additional mutations exerted a more dramatic effect on the activity of M protein by enhancing turnover of the mutant M protein. These data support the idea that mutations in M gene contribute to a delay in shut off of host transcription in cells persistently infected with VSV.

MATERIALS AND METHODS

Cells and viruses

Stocks of wild-type VSV (Indiana serotype, Orsay strain), the ts M protein mutant tsO82 (Coulon *et al.,* 1990), and the viruses from persistently infected cells were grown in BHK cells as described previously (Lyles *et al.,* 1996). TsO82 virus and the viruses from persistently infected cells were grown at 31°. Supernatants from L cells persistently infected with VSV (Youngner *et al.,* 1976) as well as the wild-type VSV used to establish the persistent infection were provided by Patricia Whitaker-Dowling (University of Pittsburgh). V6, v2, and v3 viruses were plaque isolated after infecting BHK cells with the persistent infection supernatant.

Plasmids and *in vitro* transcription

The plasmid pSV2.CAT containing the chloramphenicol acetyl transferase (CAT) reporter gene has been described (Gorman *et al.,* 1982). The plasmid vector, pSD4.2, used for *in vitro* transcription of mRNA encoding the CAT enzyme, and wt and mutant M proteins with a 3′ poly(A) tail and 5′ cap has also been described (Black *et al.,* 1994). The bacteriophage SP6 polymerase was used to transcribe CAT and M mRNAs containing 5′ caps and 3′ poly(A) *in vitro* from linearized plasmid DNA (Message Machine, Ambion, Inc.).

The M gene clones from the persistent infection population (clones A through I) were generated by infecting BHK cells with the supernatant from persistently infected cells at a multiplicity of 20 PFU/cell. Total cellular RNA was isolated 5 hr postinfection using the guanidine thiocyanate method. Viral genome RNA was reverse transcribed and amplified by polymerase chain reaction (RT-PCR) using primers specific for the M gene as described previously (Black *et al.,* 1993). PCR was carried out using the thermostable Pwo polymerase from P. woesei (Boehringer Mannheim), which contains proofreading activity. Nine separate RT-PCR reactions were analyzed to avoid preferential amplification of a limited number of M genes. Each of the nine M gene products were subcloned into the vector, pT7/T3α19 (Gibco, BRL), for sequencing.

The megaprimer method for site-directed mutagenesis (Sarkar and Sommer, 1990) was utilized to recreate the mutation at position 163 of the M protein sequence. The oligonucleotide used in the mutagenesis, 5′-GAAGAC-

CATTCGATATAGGTC-3′, was synthesized by the DNA Core Laboratory (Bowman Gray School of Medicine). Additional oligonucleotides used in this method were 5′-CCCCAAGCTTAACAGATATCACGATCTAAGTGTTATC-3′ and 5′-CCCCAAGCTTGACAGGATATTAGTTGTTCG-AGAGGC-3′ (Black *et al.,* 1993), derived from the 5′ and 3′ ends of the M gene. This mutant M gene was subcloned into the *Hin*dIII site of the pSD4.2 vector for *in vitro* transcription.

Sequencing

M gene mutants were sequenced by the dideoxy-DNA method using the Sequenase Version 2.0 T7 DNA Polymerase Sequencing kit (USB) or by automated sequencing using the ABI PRISM 377 DNA Sequencer (Perkin-Elmer). Primers used for sequencing included the 5′ and 3′ primers described above as well as those described by Coulon *et al.* (1990), which span positions 201–221 (5′-ATGATCCGCATCAATTAAG-3′) and 376–394 (5′-GT-TCTTCTAATCTAAAGGC-3′) of the M gene.

Cotransfection of M mRNA and CAT DNA or mRNA

BHK cells in 35-mm dishes were transfected with 400 ng pSV2.CAT plasmid DNA together with varying amounts of *in vitro*-transcribed M mRNA (ranging from 2 to 400 ng) and 6 μg of Lipofectin reagent (GIBCO-BRL). In experiments where CAT mRNA was transfected together with M mRNA, 20 ng of *in vitro*-transcribed CAT mRNA was used. The total amount of RNA was held constant at 1 μg by mixing M mRNA with yeast RNA (Type XI, Sigma Chemical Co.). At 5 hr posttransfection culture medium was added and cells were incubated for a total of 24 hr prior to harvesting. The methods have been described for assay of CAT activity in cell extracts by acetylation of [^{14}C]chloramphenicol and quantitation of results by radioanalytical imaging system (AMBIS Systems, Inc., San Diego), as well as expressing results in terms of relative units of CAT enzyme (Black *et al.,* 1993).

^{35}S-radiolabeling in infected and transfected cells

To analyze protein synthesis during virus infection, BHK cells were infected with wt and persistent infection viruses at a multiplicity of 20 PFU/cell in Dulbecco's minimum essential medium with 2% fetal calf serum at 31°. At 5 hr postinfection, cell monolayers were labeled with a 15-min pulse of [^{35}S]methionine (100 μci/ml) in a total of 0.5 ml of methionine-free medium and chased for several time points in DMEM plus 10% FCS. The supernatant was collected and cells were harvested. Progeny virions in the supernatant were isolated by sedimentation through a sucrose cushion (Kaptur *et al.,* 1995). Samples were lysed in SDS disruption buffer and M proteins in the virion and cell lysates were detected by separation on SDS–polyacrylamide gels and quantitated by densitometric analysis.

To determine the M protein turnover rate in transfected cells, BHK cells were transfected with 400 ng of M mRNA for 5 hr. Cells were labeled with [^{35}S]methionine (200 μci/ml) for 1 hr and chased in media lacking isotope for various times posttransfection. Cells were then harvested, and cell extracts were immunoprecipitated with the anti-M monoclonal antibody, 23H12, and processed for electrophoresis as described (Black *et al.,* 1993).

RNA synthesis in infected cells

Mouse L cells were infected with VSV (or mock infected) at an m.o.i. of 20 PFU/cell in DMEM plus 2% FCS at 37°. The virus was allowed to adsorb for 1 hr and cells were fed with medium containing 2% FCS. Parallel samples were incubated in the presence of actinomycin D (total concentration of 5 μg/ml). At 2, 4, and 6 hr postinfection, cells were labeled with [^{3}H]uridine (20 μci/ml) for 30 min, washed in phosphate-buffered saline solution, and harvested. Cells were resuspended in SDS lysis buffer containing RNase/proteinase degrader (Invitrogen) and DNA was sheared with a 20-g needle. Samples were then precipitated with 7% trichloroacetic acid on ice and washed twice with 7% trichloroacetic acid, and acid-precipitable radioactivity was measured by scintillation counting.

RESULTS

Effect of wt and mutant viruses on host RNA synthesis

The effect of infection with viruses from persistently infected cells on host RNA synthesis was determined. Individual viral clones were plaque purified from a culture supernatant from L cells persistently infected with VSV (Youngner *et al.,* 1976). This persistently infected cell line was established by coinfection with wt VSV together with defective interfering particles (Youngner *et al.,* 1976). Virus clones were derived from an assay of fluids taken at day 63 (passage 8) after the persistent infection was initiated (personal communication, Youngner *et al.,* 1976). The inhibition of host RNA synthesis by one virus clone, v6, is shown in Fig. 1. As a means of comparison, wt VSV and the VSV mutant, tsO82, were also included in this study. TsO82 virus is highly defective in its ability to induce cytopathic effects (Coulon *et al.,* 1990). L cells were infected with wt, tsO82, and v6 viruses at a multiplicity of 20 PFU/cell and labeled with [^{3}H]uridine for 30 min at 2, 4, and 6 hr postinfection. Cells were harvested and trichloroacetic acid-precipitable radioactivity was determined. Host RNA synthesis was determined by subtracting actinomycin D-resistant (i.e., viral) RNA synthesis from the total. Wt VSV effectively inhibited host RNA synthesis so that by 6 hr postinfection, only about 12% of control cellular RNA was synthesized (Fig. 1, open circles). RNA synthesis in cells infected with tsO82 virus was still about 75% of uninfected control levels at 6 hr

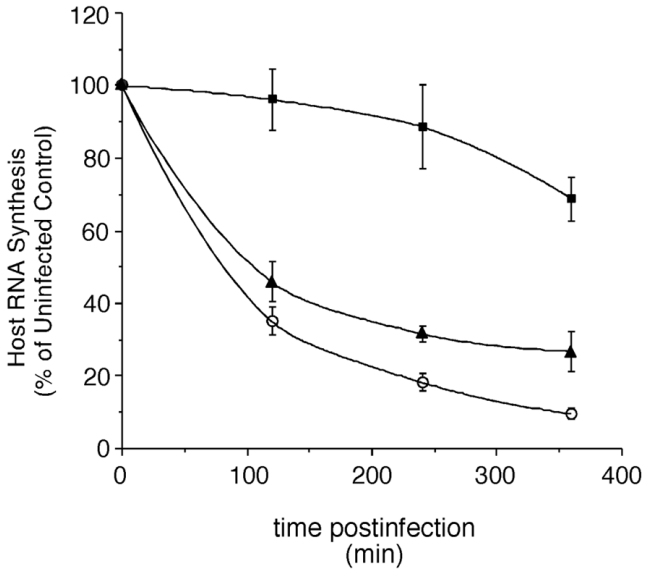


FIG. 1. Effect of wt, tsO82, and v6 viruses on host RNA synthesis. L cells were infected with wt (open circles), tsO82 (closed squares), or v6 (closed triangles) viruses at a multiplicity of 20 PFU/cell. At 2, 4, and 6 hr postinfection cells were labeled with [^{3}H]uridine (20 μCi/ml) for 30 min. Cells were lysed in SDS lysis buffer and aliquots were precipitated with trichloroacetic acid to measure acid insoluble counts. Parallel samples were incubated in the presence of actinomycin D and these values were subtracted from the total counts to determine the rate of host RNA synthesis. Data are presented as a percentage of the uninfected cell control and are means $\pm$sd for three independent experiments.

postinfection (closed squares), indicating that tsO82 virus is highly defective in inhibition of host RNA synthesis. The v6 virus isolated from persistently infected cells showed a phenotype intermediate between those of wt VSV and tsO82 viruses in that host RNA synthesis was inhibited but the inhibition was delayed relative to wt VSV (closed triangles). Results similar to those in Fig. 1 were obtained using BHK cells instead of L cells (data not shown). These results are consistent with previous data, indicating that persistent infection viruses shut off host cell RNA synthesis at a slower rate than wt VSV (Frey and Youngner, 1984). Even though viruses isolated from persistently infected cells are less effective than wt VSV in inhibition of host RNA synthesis, they are not as defective as tsO82 virus.

M proteins of viruses from persistently infected cells are expressed and turned over at rates similar to wild-type M protein

It is possible that viruses from persistently infected cells display a lag in inhibition of host RNA synthesis because they produce reduced levels of M protein due to rapid turnover, as in the case of persistent Sendai virus and measles virus infections. To test this hypothesis, the expression of M protein was examined in pulse–chase experiments. BHK cells were infected with wt or v6 viruses at a multiplicity of 20 PFU/cell. At 5 hr postinfection, cells were pulse labeled with [^{35}S]methionine for 15 min and chased in unlabeled medium for several intervals for up to 8 hr. Cells were harvested, and progeny virions in the supernatant were isolated by centrifugation through a sucrose cushion. Total cell-associated proteins were analyzed by SDS–polyacrylamide gel electrophoresis and fluorography and quantitated by densitometry. Results in Fig. 2A show labeled intracellular proteins of wt and v6 viruses at various times during the chase. Viral proteins were expressed at similar levels, although slightly lower levels were expressed in cells infected with v6 virus compared to wt VSV. Also, the synthesis of host proteins, visible as a darker background, was not shut off as effectively by v6 virus as wt VSV, as previously described (Frey and Youngner, 1984).

Figure 2B shows release of labeled viral proteins into extracellular virions at various times during the chase. The rate of incorporation of v6 viral proteins into virions was similar to that of wt VSV. Slightly lower levels of v6 virions were produced, consistent with the slightly lower levels of intracellular viral proteins. The final virus titer achieved after 18 hr infection with v6 or wt viruses was the same (2×10^9 PFU/ml).

Figure 2C shows results of densitometry of M protein in the gels in Figs. 2A and 2B. The level of M protein expression in cells infected with v6 virus at the end of the pulse ($t = 0$) was approximately 75% of that with wt VSV. Both wt and v6 M proteins were incorporated into extracellular virions at similar rates, indicating that M protein of virus from cells persistently infected with VSV is fully functional in virus replication and assembly. Similar results were obtained for two separate plaque-purified viral isolates from the same supernatant as v6 (data not shown). The results in Fig. 2C also show that the M proteins of wt VSV as well as the viruses from persistently infected cells are turned over at similar rates (half lives of intracellular M protein ranging from 150 to 186 min). Thus, there is no defect in M protein expression or virus assembly function in viruses isolated from persistently infected cells.

Sequence changes of VSV M genes from persistently infected cells

Since viruses from cells persistently infected with VSV are delayed in inhibition of host cell transcription compared to wt VSV, we wanted to determine whether sequence changes of their M genes contributed to their reduced cytopathic effect. Therefore, M genes from the heterogeneous viral pool were cloned and sequenced and tested for their ability to inhibit host gene expression in the absence of other viral components. BHK cells were infected with the supernatant from persistently infected cells or with wt VSV, and total cellular RNA was isolated. M genes were reverse transcribed using a primer complementary to viral genomic RNA and then amplified by

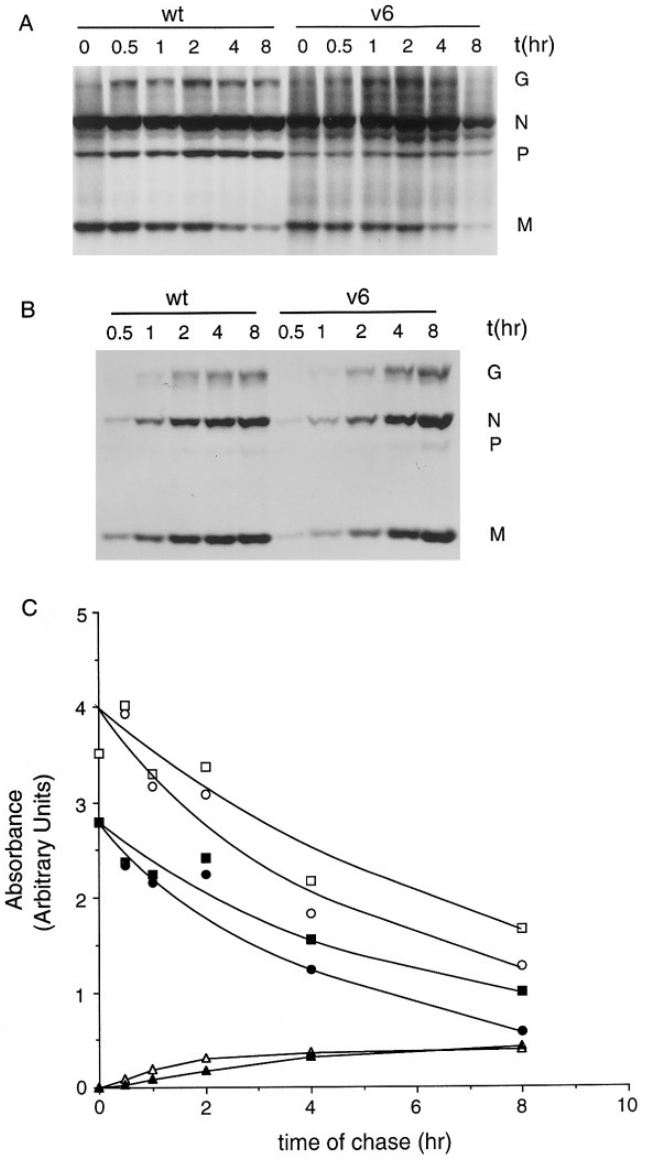


FIG. 2. Pulse–chase analysis of proteins of BHK cells during wt and v6 virus infections. BHK cells were infected with wt or v6 viruses at a multiplicity of 20 PFU/cell. Cells were pulse labeled with [^{35}S]-methionine for 15 min at 5 hr postinfection and chased in medium containing nonradiolabeled methionine for the indicated times. Cells were harvested and progeny virions in the supernatant were centrifuged through a sucrose cushion. Viral proteins in cells and virions were analyzed by SDS–polyacrylamide gel electrophoresis and fluorography. Tenfold more sample of virions (both wt and v6) were analyzed in the gel in B as compared to cell extracts in A. (A) Total protein from cells infected with wt and v6 viruses at various times during the chase. (B) wt and v6 viral proteins shed into extracellular virions during the chase. (C) Densitometry of gels in A and B. Wt M protein (open symbols) and v6 M protein (closed symbols) associated with infected cells (circles), extracellular virions (triangles), or the sum of the two (squares) are shown. These data are from one of two independent experiments that had similar results.

polymerase chain reaction. M genes from this heterogeneous viral population were cloned and sequenced. A DNA polymerase with proofreading activity (from P. woesei) was used for PCR to reduce the introduction of errors at this step. Sequences of several M gene clones derived from the wt VSV strain used to establish the persistent infection were the same as the published sequence for the M gene of the Orsay strain (Morita *et al.,* 1987). The sequences of the clones from the persistent infection population are compared to that of the wt M protein (Fig. 3). Each clone contained a single point mutation resulting in an asparagine to aspartate change at position 163 of the protein sequence (N163D). Four of the nine clones had additional mutations in their M proteins, three of which gave rise to amino acid substitutions. These data are consistent with earlier work which demonstrated the genetic heterogeneity of VSV isolated from persistently infected cells compared to wt VSV (Holland *et al.,* 1979). M gene clones were also isolated using total RNA from cells infected with v6 virus. Similar to results with the heterogeneous population, the N163D mutation was the only mutation present in all of the four clones derived from v6 virus, but several clones contained additional mutations (data not shown). None of the additional mutations shown in Fig. 3, or in the clones derived from v6, were present in more than one clone, indicating that the only consensus mutation present in the persistent population was N163D. One clone, designated N163D.2, was chosen for further study in addition to the consensus N163D mutant and wt M protein. This clone contained mutations E172K and F201S, as well as the N163D mutation.

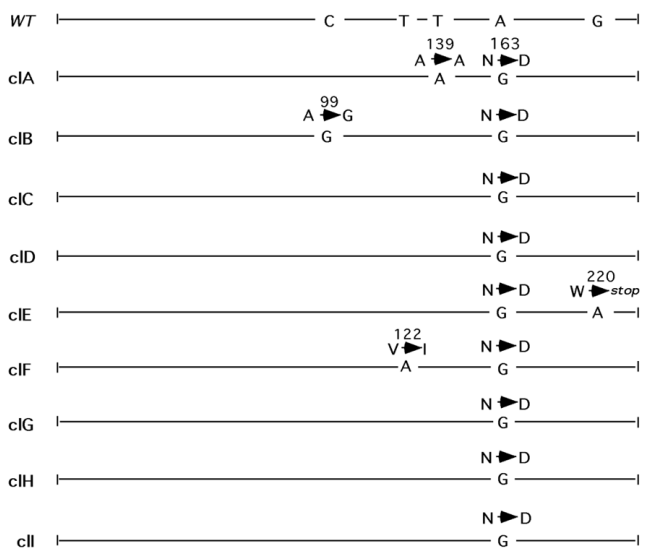


FIG. 3. M gene mutations of viruses from persistently infected cells. BHK cells were infected with wt VSV or the supernatant from persistently infected cells and total RNA was isolated. Total cellular RNA was used in nine separate reverse transcription reactions with a primer complementary to the genomic strand of VSV. Amplification by PCR was carried out using the DNA polymerase from P. woesei and M genes from each reaction were cloned and sequenced. Clones A through I are the M genes of viruses from persistently infected cells compared to the wt VSV sequence (830 bases, top). The area above each gene indicates amino acid substitutions.

Expression of mutant M proteins from transfected mRNAs

M protein inhibits its own transcription when expressed from transfected DNA vectors and therefore is difficult to express with recombinant vectors that depend on host cell transcription (Blondel *et al.,* 1990; Black *et al.,* 1992; Li and Wagner, 1988). To avoid this problem, M protein can be more efficiently expressed by transfection with *in vitro*-transcribed M protein mRNA instead of plasmid DNA (Black *et al.,* 1994). Wt, N163D, and N163D.2 M mRNAs were transcribed *in vitro* from cDNA templates in a vector containing the promoter for the SP6 bacteriophage polymerase followed by a polylinker region and a long poly(A–T) sequence. Plasmid DNA was linearized and transcribed *in vitro* with the cap analogue ^{7}mG(5′)ppp(5′)G, to generate capped, polyadenylated mRNAs. Pulse–chase experiments were used to determine the rates of synthesis and turnover of wt and mutant M proteins when expressed from transfected mRNA.

BHK cells were transfected with 400 ng of mRNA encoding wt, N163D, and N163D.2 M proteins. At 5 hr posttransfection, cells were labeled for 1 hr with [^{35}S]methionine and chased for varying times in medium containing nonradiolabeled methionine. Lysates were immunoprecipitated with an anti-M monoclonal antibody (23H12) and analyzed by SDS–polyacrylamide gel electrophoresis and fluorography (Fig. 4A). Figure 4B is a graphic representation of the data obtained by densitometry and is an average of two experiments similar to that in Fig. 4A. Data are shown as a percentage of the amount of ^{35}S-labeled wt M protein expressed immediately after the pulse ($t = 0$). N163D and N163D.2 M proteins were synthesized at a rate that was approximately 30% of wt M protein. The basis for this difference in translation rates is not understood, since the N163D M mRNA differs from wt M mRNA by a single base substitution. However, this difference was reproducible in repeated experiments that used different mRNA preparations. In these experiments, the rate of synthesis compared to wt M protein was 30.5 $\pm$ 11% (mean $\pm$ sd, $N = 4$) for N163D M protein and 32.1 $\pm$ 12% (mean $\pm$ sd, $N = 3$) for N163D.2 M protein. Wt and N163D M proteins turned over at comparable rates (half-lives of 4.2 and 3.0 hr, respectively), indicating that the consensus mutation at position 163 does not dramatically affect turnover of M protein consistent with the data in Fig. 2. However, N163D.2 M protein was turned over at a rate five times greater than wt M protein (half-life of 0.80 hr). These data indicate that the additional mutations at positions 172 and 201 confer a rapid turnover phenotype on the N163D.2 M protein.

M gene mutations affect inhibition of host gene expression

The effect of N163D M protein on host-directed gene expression was assayed and compared to that of wt M protein. A cotransfection assay was used in which M protein inhibits expression of CAT from cotransfected plasmid DNA (Black and Lyles, 1992; Black *et al.,* 1994; Lyles *et al.,* 1996). β-globin (β-glo) mRNA served as a negative control in this experiment. Wt or N163D M mRNAs were cotransfected into BHK cells at 31° together with plasmid DNA encoding the CAT reporter gene under control of the SV40 early promoter which is dependent on host factors for its transcriptional activity. At 24 hr posttransfection, cells were harvested and equivalent amounts of cell lysate were assayed for CAT activity. CAT activity was measured by the conversion of radiolabeled chloramphenicol (Cm) to its acetylated derivatives (AcCm), which were then separated by thin layer chromatography. Figure 5A is an autoradiograph from a representative experiment in which varying amounts of wt M and N163D M mRNAs were cotransfected with a constant amount of CAT DNA (400 ng). Figure 5B is a graph of data obtained by radioanalytic scanning from four separate experiments similar to that in Fig. 5A. As shown previously, expression of wt M protein inhibited CAT activity in a dosage-dependent manner (Fig. 5B, open circles). N163D also inhibited CAT enzyme expression in a concentration-dependent manner (closed triangles), but not to the same extent as wt M protein. Transfection of N163D M mRNA was about 6 to 10 times less effective than wt M mRNA in the inhibition of CAT expression. For example, in cells transfected with 4 ng of M mRNA, CAT levels were 4% of control for wt M protein versus 30% for N163D M protein. CAT activity is directly proportional to the amount of M protein expressed in this range of CAT activity (Lyles *et al.,* 1996). Therefore, when protein levels are taken into consideration, N163D was two to three times less effective than wt M protein in inhibition of CAT gene expression (6- to 10-fold less inhibition divided by 3-fold lower expression from the same amount of mRNA). This is similar to the differences in host transcription observed in cells infected with v6 versus wt VSV (Fig. 1).

In Fig. 5C, the effect of N163D and N163D.2 M proteins on CAT gene expression was tested and compared to wt and tsO82 M proteins. A larger concentration range of M mRNAs was used in these experiments compared to those in Figs. 5A and 5B. As shown before, wt M protein inhibited CAT activity in a dosage-dependent manner, whereas tsO82 M protein was highly defective in inhibition of CAT gene expression (closed squares). TsO82 M protein actually stimulated expression of CAT slightly in two of the three experiments. As in Fig. 5B, N163D M protein inhibited CAT enzyme expression but not to the same degree as wt M protein.

N163D.2 M protein (open triangles), containing two mutations in addition to the conserved N163D mutation, was highly defective in inhibition of CAT expression similar to tsO82 M protein. These results indicate that although the single mutation at amino acid 163 decreases the ability of M protein to inhibit host gene expression, additional M gene mutations from the persistent population can exert an even greater effect on the activity of M protein by enhancing the turnover of M protein.

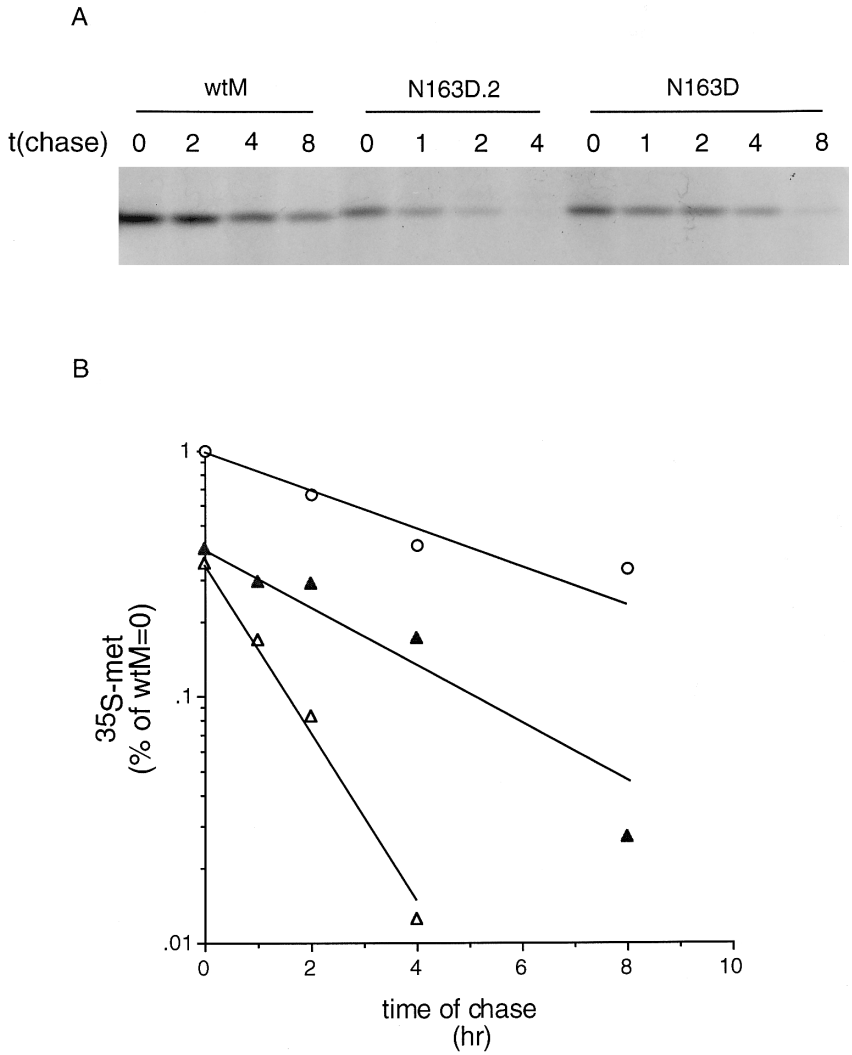
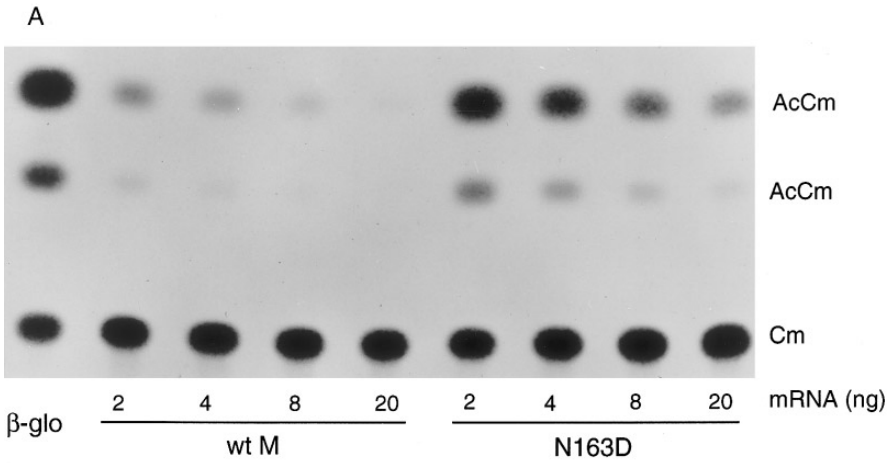


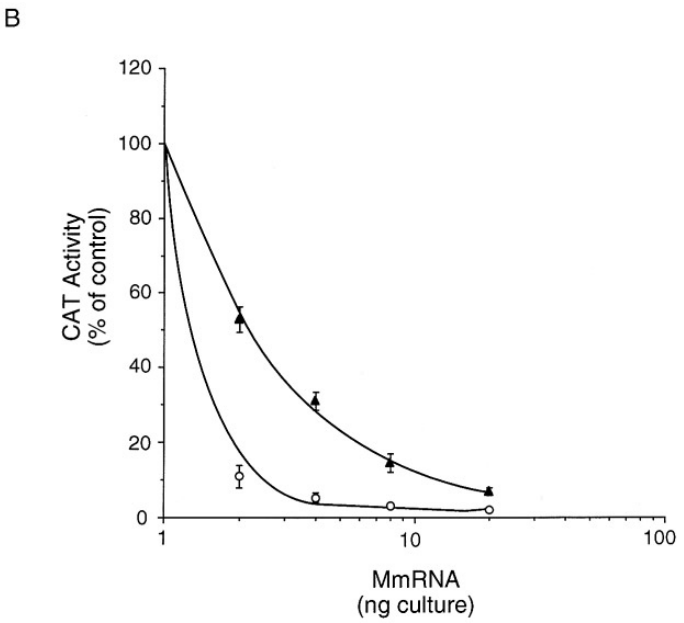
FIG. 4. Pulse–chase analysis of wt, N163D, and N163D.2 M proteins. BHK cells were transfected with 400 ng of wt, N163D, or N163D.2 mRNA for 5 hr. Cells were labeled with [^{35}S]methionine (200 μCi/ml) for 1 hr and chased in nonradiolabeled media for the indicated times. Lysates were immunoprecipitated with the anti-M monoclonal antibody, 23H12, and analyzed by SDS–polyacrylamide gel electrophoresis and fluorography (A). (B) Densitometry analysis of wt (open circles), N163D (closed triangles), and N163D.2 (open triangles) M proteins. Densitometry was carried out on two independent experiments, and data are shown as the mean radioactivity expressed as a percentage of the wt M protein level at $t = 0$.

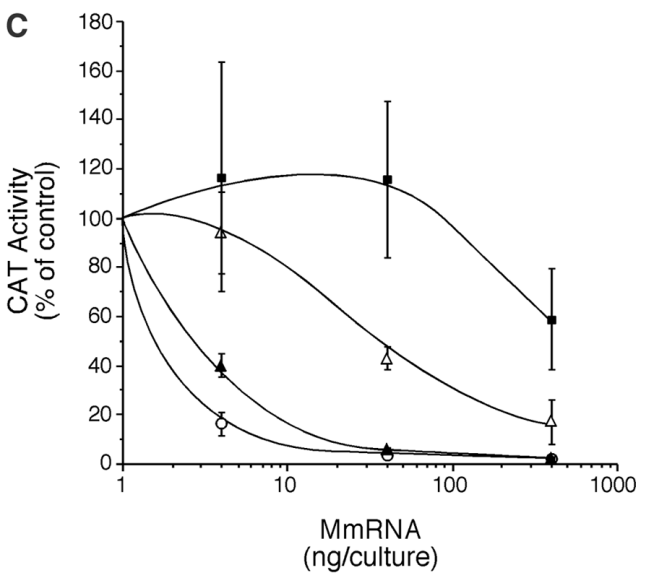
Effect of mutant M proteins on translation of cotransfected mRNAs

During VSV infection, there is a rapid inhibition of host translation, as well as shutoff of host transcription. In contrast to its effect on host transcription, M protein alone does not inhibit host translation. M protein actually stimulates translation of cotransfected mRNAs when expressed in cells independently of other viral components (Black *et al.,* 1994). To determine whether the difference in inhibition of CAT expression by wt versus N163D M proteins in Fig. 5 reflects a difference in translation of CAT mRNA, BHK cells were cotransfected with varying amounts of β-glo, wt, and N163D M mRNAs and a constant amount of CAT mRNA (20 ng). At 24 hr posttransfection, cells were harvested and CAT activity was measured as before. Results in Fig. 6 clearly indicate that wt and N163D M proteins stimulated CAT translation to

FIG. 5. Effect of wt and mutant M proteins on CAT gene expression. BHK cells (approximately 3×10^5) were transfected with 400 ng of pSV2.CAT DNA and varying amounts of *in vitro*-transcribed wt, tsO82, N163D.2, or N163D M mRNA or 400 ng of β-globin (β-glo) mRNA as a negative control. Culture medium was added at 5 hr posttransfection, and cells were incubated for a total of 24 hr at 31° prior to harvesting. CAT activity in cell extracts was assayed by the conversion of chloramphenicol (Cm) to its acetylated derivatives (AcCm) and separated by thin layer chromatography. (A) Autoradiograph of a representative experiment in which cells were cotransfected with 2, 4, 8, or 20 ng of wt or N163D M mRNA and 400 ng of pSV2.CAT DNA. (B) Quantitation of three separate experiments similar to A. CAT activity was converted to relative units of CAT (Black *et al.,* 1993) and is expressed as a percentage of the negative control cells cotransfected with β-glo mRNA. (C) Cells were transfected with 4, 40, or 400 ng of wt (open circles), N163D (closed triangles), N163D.2 (open triangles), or tsO82 (closed squares) M mRNA together with pSV2.CAT DNA.


A
AcCm
AcCm
Cm
β-glo
2
4
8
20
2
4
8
20
mRNA (ng)
wt M
N163D


B
CAT Activity
(% of control)
120
100
80
60
40
20
0
1
10
100
MmRNA
(ng culture)


C
CAT Activity
(% of control)
180
160
140
120
100
80
60
40
20
0
1
10
100
1000
MmRNA
(ng/culture)

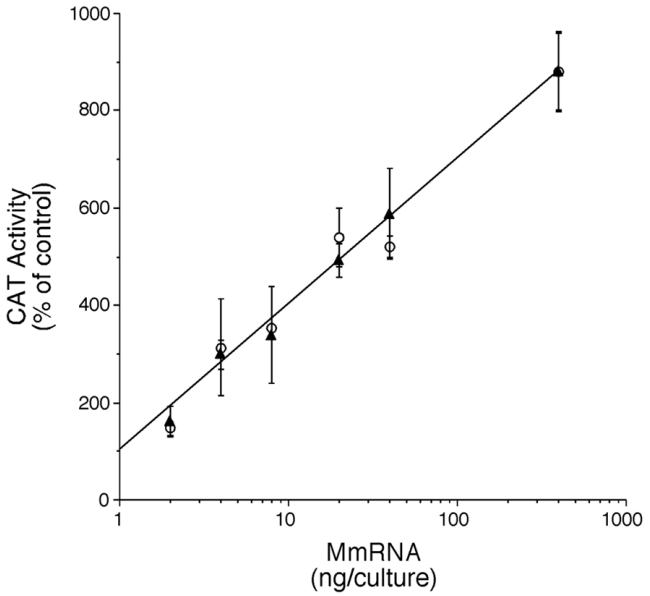


FIG. 6. Effect of N163D and wt M proteins on translation of CAT mRNA. BHK cells were cotransfected with 20 ng of *in vitro*-transcribed CAT mRNA and 2, 4, 8, 20, 40, or 400 ng of wt (open circles), or N163D (closed triangles) mRNA. Cells were harvested 24 hr posttransfection and CAT activity was measured as in Fig. 5. CAT activity is expressed as a percentage of the negative control (cells transfected with β-glo mRNA). Data shown are means $\pm$ sd for four different experiments.

similar extents with about a 2- to 5-fold enhancement in the range of 2–20 ng of M mRNA, where the data in Figs. 5A and 5B were obtained, and a 9-fold enhancement over the negative control at the highest concentration of M mRNA transfected. These data demonstrate that the difference between wt versus N163D M proteins in the inhibition of CAT gene expression observed in Fig. 5 cannot be accounted for by differences in translation of CAT mRNA.

DISCUSSION

The data presented in this study show that viruses isolated from cells persistently infected with VSV can have distinct mutations in M protein that affect its ability to inhibit host gene expression without affecting its virus assembly function. The changes in M protein were correlated with a delay in the inhibition of host-directed gene expression in virus infected cells. Previous studies have focussed on the M protein of the VSV mutant, tsO82. The tsO82 M protein contains a single substitution leading to a met to arg change at position 51 of its protein sequence (Coulon *et al.,* 1990). This single point mutation greatly decreased the ability of M protein to inhibit host cell transcription but had no effect on its ability to function in virus assembly (Black *et al.,* 1993; Kaptur *et al.,* 1995). In this paper, M genes of viruses from L cells persistently infected with VSV were cloned and sequenced. Each of the M genes from this heterogeneous population contained a substitution of asp for asn at position 163 (N163D). When recreated in the wt background and tested for its effect on host gene expression by a cotransfection assay, this mutation resulted in a phenotype intermediate between those of tsO82 and wt M proteins. Multiple factors contributed to the difference between N163D versus wt M protein in their effect on host-directed gene expression. When cells were transfected with equivalent amounts of N163D or wt M mRNA, there was a 6- to 10-fold reduction in expression of CAT from cotransfected plasmid DNA (Fig. 5). This difference could not be accounted for by differences in translation of CAT mRNA (Fig. 6). However, the N163D M protein was expressed at 3-fold lower levels than wt M protein from equivalent amounts of mRNA. This was due to a difference in rate of synthesis rather than the rate of turnover of wt versus N163D M protein (Fig. 4). Thus, inhibition of host gene expression by N163D M protein was approximately 2- to 3-fold less effective than wt M protein when the reduced expression levels were taken into account. This is similar to the differences in host transcription in cells infected with v6 virus versus wt VSV (Fig. 1). This difference between wt and N163D M proteins is greater than would be expected from typical strain variation in M protein sequence. For example, the M proteins of the San Juan and Orsay strains of VSV differ by 5 amino acid substitutions at regions scattered throughout the protein (Morita *et al.,* 1987). Two of the strain differences (at M protein positions 54 and 171) are near the sites of the mutations in tsO82 and N163D M proteins, respectively. However, the M proteins of the San Juan and Orsay strains are indistinguishable in their ability to inhibit CAT expression in cotransfection assays (Black *et al.,* 1993; and unpublished data). This indicates that only specific types of mutations are responsible for the reduced ability of M protein to inhibit host gene expression. The results from cotransfection assays were correlated with the levels of inhibition of host RNA synthesis in cells infected with the persistent infection viruses, tsO82 virus and wt VSV. These data support the idea that specific mutations in M protein contribute to defective shut off of host transcription in cells infected with VSV mutants.

Wt M protein stimulates translation of cotransfected mRNAs, *in vivo,* in the absence of other viral proteins (Black *et al.,* 1994; and Fig. 6). M protein's ability to enhance host translation is genetically correlated with M protein-mediated inhibition of host transcription and may reflect a decrease in competition between the transfected mRNA and endogenous host mRNA. For example, tsO82 M protein is highly defective in inhibition of host-directed transcription and also fails to stimulate translation of cotransfected CAT mRNA (Black *et al.,* 1994). Results from experiments presented here showed that N163D M protein enhanced CAT translation similarly to wt M protein (Fig. 6), even though it was less effective than wt M protein in its overall ability to inhibit host gene expression. Translation of host mRNAs is severely inhibited by VSV infection. Several VSV mutants containing M gene mutations, including tsO82 as well as

the persistent infection viruses, are defective or display a lag in shutoff of host translation (Francoeur *et al.,* 1987; Frey and Youngner, 1984). The delay in inhibition of host translation was also demonstrated with the v6 virus from persistently infected cells (Fig. 2A). Therefore, these results reinforce the idea that during VSV infection, the inhibition of host translation may be quite complex and requires additional or separate viral gene products besides M protein.

A characteristic of persistent RNA virus infections is that the viruses evolve rapidly giving rise to a heterogeneous population. The observation that M gene sequences from a persistent VSV population are variable has been shown previously by Holland and coworkers. By oligonucleotide mapping of the RNA genome they demonstrated that their persistent infection virus population in BHK cells was undergoing continuous and extensive mutational change over time (Holland *et al.,* 1979). In contrast, under low multiplicity passage conditions, the consensus T1 fingerprints of wt VSV remained unchanged over multiple passages unless the equilibrium was disrupted by mutagenesis or high multiplicity passages which select for DI particles (Steinhauer *et al.,* 1989). These observations demonstrated the importance of selective pressure, such as the conditions necessary to establish persistent infections, in the evolution of RNA virus population. In our work, we have shown by direct sequence analysis that M genes from a pool of persistent infection viruses are genetically heterogeneous. Each of the nine M gene clones sequenced contained the consensus mutation at position 163 (N163D). However, four of nine of them contained additional mutations revealing the genetic diversity of the population. The occurrence of M gene mutations in cells persistently infected with VSV (Fig. 3) was consistent with the misincorporation rates for several different genomic sites of VSV, including the M gene, which have been estimated to be on the order of 10^{-3} to 10^{-4} as determined by T1 oligonucleotide mapping (Steinhauer *et al.,* 1989). The genetic heterogeneity of the persistent VSV population has the potential to generate additional mutants that are more defective than the consensus sequence.

The N163D.2 M protein mutant, which contains two additional mutations in its carboxy terminal region, was highly defective in inhibition of CAT activity and was turned over more rapidly than wt and N163D M proteins. Clearly, these additional mutations had a more dramatic effect on the activity of M protein than the N163D mutation. The faster turnover of N163D.2 M protein may play a role in the decreased cytopathic activity of the protein but cannot entirely account for this change since N163D M protein is turned over at rates similar to wt M protein and still has a reduced effect on inhibition of host transcription. However, the enhanced turnover of the N163D.2 M protein is probably a major factor in its defectiveness compared to the N163D mutation alone. This enhanced turnover is reminiscent of persistent measles and Sendai virus infections which are associated with unstable matrix proteins (Cattaneo *et al.,* 1988; Roux and Waldvogel, 1982; Young *et al.,* 1985). In these systems, it is believed that the high turnover rate due to multiple M protein mutations is associated with the maintenance of persistent infections by reducing virus yields (Cattaneo *et al.,* 1988; Roux and Waldvogel, 1982). Even though persistent VSV infection is capable of giving rise to M proteins that are rapidly turned over (i.e., the N163D.2 mutant), such mutants are not selected as consensus sequences. Instead, the consensus N163D mutation in VSV M protein found in persistently infected cells reduced the potency of M protein in inhibition of host gene expression without affecting its function in virus assembly. Therefore, in persistent VSV infections, mutations in M may be selected primarily to decrease the overall cytopathic effect allowing survival of both virus and host. Similar to the case of VSV infections, in reoviruses, the σ3 outer capsid polypeptide, which is encoded by the S4 dsRNA segment, is responsible for inhibiting cell protein and RNA synthesis (Sharpe and Fields, 1982). Using a genetic approach, it was found that mutations in the reovirus S4 gene play an important role in the establishment of persistent infections in L cells, and that the low virulence and attenuation of high passage stocks of reovirus are due to mutations in the σ3 protein (Ahmed and Fields, 1982).

The establishment of the persistent state involves multiple genetic alterations in many viruses, including VSV. It has been shown that in two persistently infected cell lines initiated with VSV, the genomic 3′ terminal region contained several sequence mutations (Wilusz *et al.,* 1985). It was speculated that mutations in the L gene may also play a role in the persistent state since viruses isolated from persistently infected cells displayed a phenotype of reduced viral RNA synthesis and accumulate temperature-sensitive mutations in their L genes (Frey and Youngner, 1984). For some viruses, mutations in both the host cells as well as the viruses are selected during the establishment of persistent infections (Dermody *et al.,* 1993; Chen and Baric, 1995; de la Torre *et al.,* 1988; Ron and Tal, 1985). In the case of VSV, genetic changes in the host cell during persistent infections have not been fully explored. However, the viral mutations are clearly important, since viruses from persistently infected cells can be reintroduced into other cell lines and readily establish new persistent infections (Youngner *et al.,* 1978; Stanners *et al.,* 1977).

In summary, the data presented in this paper suggest that in a cell line persistently infected with VSV, the virus has evolved strategies to allow it to maintain itself in its host. Viruses from persistently infected cells have a reduced ability to inhibit host RNA and protein synthesis. Furthermore, mutations in the M proteins of viruses from these persistently infected cell lines contribute to the decreased cytopathic activity. However, their cytopathic activity has not been completely inactivated by mutations, which might otherwise lead to elimination of the virus from the host as a result of anti-viral defense mechanisms. During the development of the persistent state, mutations are incorporated throughout the viral genome and it is likely that for persistent VSV infections, M protein mutations that decrease the cytopathic effect of the virus

work in concert with other viral mutations to allow persistence of the virus in its host.

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